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(54) Title: METHOD OF MODULATING MICROCIRCULATION

(57) Abstract

The present invention provides, *inter alia*, use of L-arginine, a precursor of L-arginine and/or physilogically acceptable salts thereof, or of (i) a nitric oxide donor, and/or (ii) a substrate of the nitric oxide synthetase, and/or (iii) a precursor of the said substrate, in the preparation of a medicament or nutritional formulation for the amelioration of micro-circulatory hypo-perfusion, and/or the treatment or prophylaxis of hypoperfusion-reperfusion injury, in patients which have undergone elective surgery, characterized in that the medicament or nutritional formulation is pre-operatively administered to the patient.

METHOD OF MODULATING MICROCIRCULATION

The present invention relates to a method of modulating the micro-circulation, particularly in patients who have undergone, or are due to undergo, elective surgery.

Disruption of the microvasculature is a key factor in mechanisms of *inter alia* hepatic, mesenteric and cardiac ischemia-reperfusion injury. Recent *in vitro* studies using a blood free, low-flow, reflow liver perfusion model have demonstrated that reperfusion injury can be reduced by improving the microcirculation. For example, perfused livers from rats pre-treated with a fish-oil diet showed a marked improvement in microcirculation and a significant reduction in hepatic damage. In addition, it is also known that adenosine, a known vasodilator and an essential component in Caroline Rinse solution, improves survival following liver transplantation.

The present invention provides *inter alia*, a medicament, pre-operative administration of which to patients due to undergo elective surgery has a prophylactic effect vis-à-vis hypoperfusion-reperfusion injury in numerous micro-circulatory systems.

According to the present invention there is provided the use of L-arginine, a precursor of L-arginine and/or physiologically acceptable salts thereof, in the preparation of a medicament or nutritional formulation for the amelioration of micro-circulatory hypo-perfusion, and/or the treatment or prophylaxis of hypoperfusion-reperfusion injury, in patients which have undergone elective surgery, characterized in that the medicament or nutritional formulation is pre-operatively administered to the patient.

Preferred precursors of L-arginine are either ornithine or glutamine, particularly preferred ornithine.

The present invention further provides a method for the amelioration of micro-circulatory hypo-perfusion, and/or the treatment or prophylaxis of hypoperfusion-reperfusion injury, in patients which have undergone elective surgery, characterized in that L-arginine, a precursor of L-arginine and/or physiologically acceptable salts thereof is pre-operatively administered to the patient.

L-arginine is the substrate for nitric oxide synthetase (NOS), the enzyme responsible for the production of nitric oxide, a highly unstable molecule which *inter alia* mediates smooth muscle

or formulation is conveniently administered in the form of an aqueous liquid. The medicament or formulation in a form suitable for enteral application is accordingly preferably aqueous or in powder form, whereby the powder is conveniently added to water prior to use. For use in tube feeding, the amount of water to be added will depend, *inter alia*, on the patient's fluid requirements and condition.

The beneficial effect of the use of the medicament or formulation of the present invention for treatment or prophylaxis of hypoperfusion-reperfusion injury, in patients which have undergone elective surgery is due to improvement of the microcirculation in the respective organs.

Greatest improvements in micro-circulations are associated with the mesenteric, gut, hepatic and cardiac circulations accrue from use of the medicament or formulation according to the invention.

The amount of medicament or formulation to be administered depends to a large extent on the patient's specific requirements. In the case that the medicament or formulation comprises L-arginine (or a pharmaceutically acceptable salt thereof) or a precursor of L-arginine, such as ornithine, the patient should be administered enough to increase the plasma total concentration of L-arginine from basal levels of about 70-85μM to about 100-200μM, preferably to about 120-150μM. The plasma total concentration of L-arginine should preferably not be elevated to above about 200μM, as a consequence of use according to the invention. The medicament or formulation may be so formulated as to deliver to the patient about 1 to about 30g, preferably 5 to 18g, of nitric oxide synthetase substrate or L-arginine, a precursor of L-arginine and/or physiologically acceptable salts thereof, per 24 hours, or about 0.1 to about 20g of nitric oxide donor per 24 hours. It will be appreciated, however, that in particular where the medicament or formulation comprises nitric oxide donors *per se*, the patient should not be administered so much medicament or formulation that the (cyto)toxic effects of nitric oxide become apparent.

It is particularly preferred that the substrate of the nitric oxide synthetase is L-arginine or a physiologically acceptable salt thereof. In a non endotoxin/cytokinin stressed individual it is to be expected that the substrate is utilized by the calcium and NADPH dependant/calmodulin sensitive constitutive nitric oxide synthetase and accordingly, the invention contemplates this form of the synthetase as the target for the L-arginine contained within the medicament or formulation, or the L-arginine which results from the precursor (such as ornithine or glutamine

hospitalized (up to 20 days), if not longer.

In a preferred embodiment the present invention provides the use of L-arginine, a precursor of L-arginine and/or physiologically acceptable salts thereof together with omega-3 PUFAs which are optionally protected in a pharmacologically acceptable way against peroxidation, in the preparation of a medicament or nutritional formulation for the amelioration of micro-circulatory hypo-perfusion, and/or the treatment or prophylaxis of hypoperfusion-reperfusion injury, in patients which have undergone elective surgery, characterized in that the medicament or nutritional formulation is pre-operatively administered to the patient.

In a further preferred embodiment the present invention provides the use of (i) a nitric oxide donor, and/or (ii) a substrate of the nitric oxide synthetase, and/or (iii) a precursor of the said substrate together with omega-3 PUFAs, in the preparation of a medicament or nutritional formulation for the amelioration of micro-circulatory hypo-perfusion, and/or the treatment or prophylaxis of hypoperfusion-reperfusion injury, in patients which have undergone elective surgery, characterized in that the medicament or nutritional formulation is pre-operatively administered to the patient.

It is particularly preferred that the substrate of the nitric oxide synthetase is L-arginine or a pharmaceutically acceptable salt thereof.

The medicament of formulation used according to the invention may (and preferably will) still further comprise other nutritionally advantageous components such as vitamins, minerals, trace elements, fibers (preferably soluble fibers) as well as nitrogen sources, carbohydrate sources and additional fatty acid sources.

Examples of suitable nitrogen sources include nutritionally acceptable proteins such as soy bean or whey derived proteins, caseinates, and/or protein hydrolysates. Suitable carbohydrate sources include sugars such as maltodextrins. Examples of suitable fatty acid energy supply sources include triglycerides, as well as di- and monoglycerides.

Examples of vitamins suitable for incorporation into the medicament of the invention include Vitamin A, Vitamin D, Vitamin K, folic acid, thiamin, riboflavin, Vitamin B_6, Vitamin B_{12},

Example 1 ***"low-flow reflow of liver perfusion - animal model"***

Animals and diets

Male Sprague-Dawley rats weighing between 100-160g are caged individually and given powdered diets containing 5% of weight as corn oil, encapsulated fish oil, or encapsulated fish oil with additional L-arginine in a blind design *ad libitum* for 12-19 days or a powdered diet containing 5% of weight as L-arginine for three days (Table 1). The diets are kept under nitrogen at 4°C, and fresh diets are provided daily. Food intake is assessed by weighing the diet remaining each day. Rats are fasted for 24h prior to liver perfusion.

Perfusion

Rats are anaesthetized with pentobarbital sodium (1µl/g) before surgery and livers are removed surgically and perfused, *via* a cannula inserted into the portal vein, with Krebs-Henseleit bicarbonate buffer (pH 7.4, 37°C) saturated with an oxygen-carbon dioxide (95:5) mixture in a non-recirculating system as is known in the art. After surgery, livers are perfused at flow rates of about 1ml/g/min for 75 minutes (low-flow). Under these conditions, periportal areas are normoxic while adjacent pericentral regions are anoxic. Subsequently, livers are perfused at normal flow rates (4 ml/g/min) for 40 minutes (reflow). Oxygen concentration in the effluent perfusate is monitored continuously with a Teflon-shielded, Clark-type oxygen electrode. Oxygen uptake is calculated from the influent minus effluent concentration difference, the flow rate and the liver wet weight.

A three way stop-cock is inserted into the tubing just prior to the cannula entering the portal vein. A polyethylene tube (PE 240) is placed in the three way stop-cock perpendicular to the cannula entering the portal vein. Portal pressure is monitored by changes in the height of a water column during perfusion. The common bile duct is cannulated with polyethylene tubing (PE-10; Clay Adams), and aliquots of bile are collected into tarred vials at 15-min intervals in the low-flow and at 10-min intervals during reflow periods. Rates of bile production are calculated from weight of bile, time intervals and the wet weight of the liver.

Assays

Lactate dehydrogenase (LDH) activity in the perfusate is determined using standard enzymic techniques, and Malondialdehyde (MDA) is assessed using thiobarbituric acid according to

respectively ($p>0.05$, student's t-test). Taken together, reperfusion injury, which occurs when oxygen is re-introduced into previously anoxic liver, is minimized by pre-feeding rats a diet supplemented with fish-oil and arginine.

Effect of Arginine pre-feeding on malondialdehyde production

MDA, an end product of lipid peroxidation, is released into the effluent perfusate at rates around 15 nmol/g/h during 75 minutes of low-flow perfusion in livers from corn oil-fed, encapsulated fish-oil-fed, L-arginine and encapsulated fish-oil plus arginine fed rats. When flow rates were restored to normal, MDA production increased rapidly to peak values in about 15 minutes and then decreased slightly. Maximal MDA production during the reperfusion period was around 90 nmol/g/h in control rats, 80 nmol/g/h in encapsulated fish-oil-treated rats, 45 nmol/g/h in L-arginine-treated rats and 67nmol/g/h in rats pre-fed encapsulated fish oil supplemented with arginine, respectively, the differences between the various groups not being statistically significant.

Effect of Arginine pre-feeding on trypan blue distribution time

Trypan blue distribution time is an indicator of the hepatic microcirculation. It took 10.7 minutes for trypan blue to distribute evenly in livers from corn-oil-treated rats, 6.0 minutes in the case of rats pre-fed with encapsulated fish-oils, 3.8 minutes in the case of rats pre-fed with L-arginine and 2.8 minutes for rats pre-fed with encapsulated fish-oils supplemented with arginine. These results are extremely significant ($p<0.05$, Student's T Test).

The above description (the results of which are summarized in Table 2) clearly indicates that pre-feeding with an arginine rich diet provides for an improved micro-circulation in organs likely to be subject to hypoperfusion-reperfusion injury, and that this protective effect of arginine is apparent even though the diet is curtailed prior to surgery.

One of the consequences of elective surgery (as well as accident surgery for that matter) is a partial shut-down of the micro-circulations associated with the liver and heart, but particularly the mesentery/gut. This "shut-down" facilitates metabolic changes associated with such micro-circulations which provides for free radical damage, particularly by superoxide anions, upon their subsequent reperfusion. Such free radical damage may render the gut microcirculation leaky to products of digestion, in particular bacterial cell components. Such components may enter the

The mineral mixture comprises:

Mineral		
Calcium	% w/w diet	0.50
Chloride	% w/w diet	0.05
Magnesium	% w/w diet	0.04
Phosphorus	% w/w diet	0.40
Potassium	% w/w diet	0.36
Sodium	% w/w diet	0.05
Sulfur	% w/w diet	0.03
Chromium	mg/kg diet	0.30
Copper	mg/kg diet	3.00
Fluoride	mg/kg diet	1.00
Iodine	mg/kg diet	0.15
Iron	mg/kg diet	35.00
Manganese	mg/kg diet	50.00
Selenium	mg/kg diet	0.10
Zinc	mg/kg diet	12.00

The vitamin mixture comprises per kg diet:

$A^{(1)}$	4 000.00 IU
$D^{(2)}$	1 000.00 IU
$E^{(3)}$	30.00 IU
K_1	50.00 µg
Choline	1 000.00 mg
Folic acid	1.00 mg
Niacin	20.00 mg
Pantothenate (calcium)	8.00 mg
Riboflavin	3.00 mg
Thiamin	4.00 mg
Vitamin B_6	6.00 mg
Vitamin B_{12}	50.00 µg

(1) Vitamin A: 1 IU = 0.500 µg retinol
(2) Vitamin D, 1 IU = 0.025 µg ergocalciferol
(3) Vitamin E, 1 IU = 1mg DL-α-tocopheryl acetate.

Example 2 ***"Patient Study"***

To evaluate whether preoperative administration of a supplemented enteral formula (see Table 3) results in an improvement of the microcirculation in patients undergoing to elective major abdominals surgery, intraoperative mesenteric blood flow (with doppler) at laparotomy and at the end of surgery, and intraoperative mucosa pHi and oxygenation (tonometry) at laparotomy and at the end of surgery were measured. Monitoring of the gastrointestinal mucosal perfusion by tonometry during major surgery and early after trauma appear to be a very sensitive method to predict the developments of organi failure and poor outcome. It has been repeately shown that patients with low postinjury pHi have high risk of morbidity and mortality.

The study included 40 patients who were be submitted to radical surgery for gastric, pancreatic and colorectal cancer. Patients were randomised in two groups: Group A - which received preoperative enteral supplemented formula via the oral route for 7 days before surgery plus postoperative nutrition from the end of operation for 7 days and Group B - which received a preoperative enteral control diet via a oral route for 7 days before surgery plus postoperative nutrition from the end of operation for 7 days. The composition of the diets is reported in Table 3.

Patients drank 1 liter of either supplemented enteral formula or control formula per day (for 7 days before surgery), corresponding to 1000 kcal/day, and they were allowed to eat a standard diet contemporaneously. Postoperatively the two groups received the same energy (25 kcal/kg/day) and nitrogen (0.25 g N/kg/day) intake.

Plasma arginine levels 1 day before surgery were at about 65 $\pm$ 20 µmol/l for Group B and at about 105 $\pm$ 46 µmol/l for Group A.

Table 5 / Postoperative Tonometry, measurement of intestinal mucosa oxygen metabolism pHi:

Diet	Day 1	Day 4	Day 7
Supplemented	7.39±0.23*	7.41±0.16*	7.40±0.12
Standard	7.33±0.18	7.36±0.21	7.38±0.10

* $p<0.05$ vs. Control

Although an adequate blood flow is no guarantee of a good tissue oxygen tension, delivery and utilization, the date given above shows that a higher intestinal microperfusion, as directly measure by laser Doppler flowmetry technique, paralleled a better gut mucosal oxydative metabolism.

Whilst the invention has been particularly described with respect to the above specific examples, the skilled man will understand that the invention is not limited to this but includes all logical developments.

For example, the invention further provides the use of L-arginine, a precursor of L-arginine and/or physiologically acceptable salts thereof, or of a nitric oxide donor, and/or a substrate of the nitric oxide synthetase, and/or a precursor of the said substrate in the preparation of a medicament or nutritional formulation for the prevention and/or reduction of neutrophil activation or adherence, or the prevention and/or reduction of superoxide anion mediated free radical damage, in patients which have undergone elective surgery, characterized in that the medicament or formulation is pre-operatively administered to the patient.

8. Use according to any preceding claim, wherein the nitric oxide substrate is L-arginine or a physiologically acceptable salt thereof.

9. Use according to any preceding claim, wherein the precursor is ornithine or glutamine, which upon ingestion/digestion by the patient is metabolized into L-arginine

10. Use according to any preceding claim, wherein the donor is selected from the group consisting of glycerol trinitrate, isosorbide dinitrate, nitroprusside, 8-bromoguanosine-3,5'-monophosphate, spermine-NO, spermidine-NO, and SIN1.

11. Use according to any one of the preceding claims, characterized in that the medicament or formulation further comprises at least one compound selected from the group consisting of superoxide free radical scavengers, angiotensin converting enzyme (ACE) inhibitors, non-steroidal anti-inflammatory compounds, omega-3 polyunsaturated fatty acids which are protected in a pharmacologically acceptable way against peroxidation, vitamins, mineral elements, soluble fibre, caseinates or protein hydrolysates and omega-6 polyunsaturated fatty acids.

12. Use of L-arginine, a precursor of L-arginine and/or physiologically acceptable salts thereof, a nitric oxide donor, and/ or a substrate of the nitric oxide synthetase, and/or a precursor of the said substrate, in the preparation of a medicament for the prevention or reduction of neutrophil activation and/or adherence, or the prevention or reduction of superoxide anion mediated free radical damage, in patients which have undergone elective surgery, characterized in that the medicament or formulation is pre-operatively administered to the patient.

13. Use according to any one of the preceding claims, characterized in that the medicament or formulation further comprises omega-3 polyunsaturated fatty acids which are optionally protected in a pharmacologically acceptable way against peroxidation.

INTERNATIONAL SEARCH REPORT

Int. ..ional Application No
PCT/EP 96/02124

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CIRCULATION, vol. 89, no. 4, 1994, pages 1254-61, XP000575704 RICHARD ET AL: "Ischemic preconditioning protect against coronary endothelial dysfunction induced by ischemia and reperfusion" * abstract *	1-13
Y	US,A,5 385 940 (M.A. MOSKOWITZ) 31 January 1995 * col.2, l.17-24; claims 1-3 *	1-13
Y	NEW HORIZONS, vol. 3, no. 1, February 1995, pages 105-12, XP000575669 D.J.LEFER: "Myocardial protective actions of nitric oxide donors after myocardial ischemia and reperfusion" *Abst.; p.109, right hand col., l.12-20; p.110, left hand col., l.2-right hand col., bot.* see figure 1	1-13
Y	THE J. OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS, vol. 260, no. 2, 1991, pages 668-75, XP002011076 SIEGFRIED ET AL: "Cardioprotection and attenuation of endothelial dysfunction by organic nitric oxide donors in myocardial ischemia-reperfusion" * abstract; p.674, right hand col., l.30-45 *	1-13
A	WIENER MEDIZINISCHE WOCHENSCHRIFT, vol. 143, no. 7-8, 1993, pages 148-58, XP000576791 MENGER ET AL: "Die Mikrozirkulation des Skelettmuskels nach Ischämie und Reperfusion" * Abstract *	1-13